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IMMUNOLOGICAL EVIDENCE THAT PLASMA-MEMBRANE 5'-NUCLEOTIDASE IS A TRANSMEMBRANE PROTEIN

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Antibodies inhibiting specifically plasma membrane-bound 5'-nucleotidase were used to determine the disposition of the enzyme in various lymphoma cell lines. Fluorescent Fab fragments of the inhibiting antibodies bound to MF_2s and MOPC 173 plasmacytoma cells, whereas no fluorescence was observed with P 1798 thymoma cells in which the enzyme was absent. The relative potency of the antiserum in inhibiting the enzyme in right-side-out and inside-out plasma membrane vesicles prepared from the above cell lines indicated that various groups of antigenic determinants were present. One group of antigenic determinants was present at the external face of the cell, and a second was associated with the inner surface of the membrane. A third group of antigenic determinants was located in the vicinity of the active site of the enzyme and it is this group that varied in the various plasmacytoma cells studied. The results are interpreted as immunological evidence that 5'-nucleotidase is a transmembrane glycoprotein.

Introduction

Although extensively studied, the plasma-membrane enzyme 5'-nucleotidase (EC 3.1.3.5) hydrolyzing 5'-ribonucleotides to ribonucleosides has not yet been unambiguously assigned to any known cellular function. The hydrolytic activity is generally located at the external face of the plasma membrane of several cell types, including hepatocytes [1,2] and lymphocytes [3–5]. Enzymic activity is unevenly distributed among the lymphocyte subpopulations studied [5–7]. For example, we have shown [8] that murine plasmacytomas (representative of B cells) and thymomas bearing Lyt-1 antigens (T-helper-type cells) exhibit 5'-nucleotidase activity, whereas thymomas bearing Lyt-2 and Lyt-3 antigens (T-precursor-type and T-cytotoxic/suppressor type cells) lack the enzymic activity. Furthermore, a nucleoside transporting function correlates with activity hydrolysing 5'-ribonucleotides in lymphocytes [9,10] and heart [11].

To fulfill a transport function in the plasma membrane, a transmembrane orientation for the 5'-nucleotidase would be expected, a possibility already supported by the demonstration that the enzyme is a glycoprotein [4,12], with its hydrolytic site exposed to the extracellular space. However, in an MF_2s cell line, the hydrolytic site of the enzyme was located at the cytoplasmic face of the plasma membrane [13]. In the present paper, we take advantage of the varying disposition of the enzyme in murine plasmacytoma cell plasma membranes to present evidence, using specific inhibitory antibodies, that 5'-nucleotidase possesses antigenic sites exposed on both the inner and outer face of the plasma membrane. Thus, 5'-nucleotidase can be classified along with other transport proteins as being transmembrane.

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Abbreviation: FITC, fluorescein isothiocyanate.

Materials and Methods

Cells. MOPC 173 murine plasmacytoma cells, its derived cell line MF_2s and P 1798 murine thymoma cells were adapted to grow as ascites in Balb/c mice.

Plasma membrane isolation. Plasma membranes were isolated from MF_2s and MOPC 173 cells as described in Ref. 8. The purified membranes from MF_2s and MOPC 173 were harvested from the lightest fraction of a discontinuous sucrose gradient at a density 1.14–1.12. The purification of the 5′-nucleotidase was between 10- and 15-fold compared to the cell lysate activity, while it was only 2-fold at d 1.16, and no purification was found in other layers of higher density. Moreover, marker enzymes characteristic of other cell organelles (e.g., mitochondria, endoplasmic reticulum and lysosomes) were absent or present at low activities in this fraction. Right-side-out and inside-out vesicles were separated as described in Ref. 14.

Determination of sialic acid. The total amount of sialic acid was estimated after acidic hydrolysis by the Warren technique [15]; specific measurements of exposed sialic acids were made by adding *Clostridium perfringens* neuraminidase attached to beaded agarose (EC 3.2.1.18; Sigma Chemical Co.).

Absorption of antiserum. A rabbit antiserum against detergent-extracted mouse liver plasma membranes, in which the major constituent was 5′-nucleotidase [15], was prepared as described previously [16]. Absorbed antiserum was prepared by incubating overnight at 4°C 100 μl of serum together with $40 \cdot 10^6$ MOPC 173 or MF_2s cells or with $120 \cdot 10^6$ P 1798 cells. These suspensions were then centrifuged to pellet the cells and the supernatants were absorbed a second time under the same conditions. Antiserum was also absorbed with inside-out vesicles prepared from MF_2s plasma membranes (100 μl of serum was mixed with vesicles [250 μg protein]).

FITC-Fab fragments of these antibodies were prepared by coupling an immunoglobulin G fraction with fluorescein isothiocyanate (FITC) as described in Ref. 17. Pepsin treatment was used to prepare the Fab fragments.

Enzymatic assays. 5′-Nucleotidase was assayed in a 40 mM Tris-HCl buffer, pH 7.5/100 mM NaCl. The reaction was started by addition of 5′AMP (1 mM final concentration) and the activity determined as in Ref. 8. Other enzymatic activities were assayed as described in Ref. 8, to check plasma membrane purity and the specificity of the antiserum. Protein was determined by the method of Lowry et al. [18] using bovine serum albumin as standard.

Materials. All chemical reagents were of analytical grade and were obtained from Sigma Chemical Co. or Merck. Silica-distilled water was used throughout.

Results

It was previously shown by measuring 5′-nucleotidase activity during cell lysis that, while MF_2s exhibited hydrolytic activity at the inner face of the plasma membrane, MOPC 173 had this activity at the cell surface [8].

Right-side-out and inside-out vesicles prepared from MF_2s had the same sialic acid lipid and protein composition (data not shown) but differed in the following characteristics: (i) 90% of sialic acid was released from right-side-out vesicles by neuraminidase-biogel in about 10 min; none was released from inside-out vesicles after 30 min. (ii) Concanavalin A binding assayed with [^{3}H]acetyl concanavalin A was located exclusively on right-side-out vesicles. (iii) 5′-Nucleotidase and $(Na^+ + K^+)$-ATPase activities in inside-out vesicles were 3-fold those found in right-side-out vesicles. Inside-out and right-side-out vesicles exhibited the same activities after permeabilisation, either by deoxycholate (0.1%) or Triton X-100 (1%). (iv) A 350-fold increase of the $(Na^+ + K^+)$-ATPase sensitivity to ouabain was shown upon EDTA treatment of inside-out, but not right-side-out, vesicles.

Specificity of the serum

The antiserum was shown using liver plasma membranes to inhibit 5′-nucleotidase in both membrane-bound and in soluble forms; furthermore, phosphodiesterase and nucleotide pyrophosphatase activities and insulin binding were unaffected by the antiserum [12]. In the present work the antiserum had no effect on nonspecific phosphatases, $(Na^+ + K^+)$-ATPase and Mg^{++}-ATPase activities of lymphoma plasma membranes.

Examination of murine thymomas and plasmacytomas has shown that some cell lines lack 5′-nucleotidase activity [8]. Using fluorescent FITC-Fab fragments of anti-5′-nucleotidase antibodies, we

observed that cells devoid of the enzyme activity (e.g., P 1798 thymoma) did not fluoresce, whereas cells exhibiting 5'-nucleotidase activity (e.g., MF_2s or MOPC 173 plasmacytoma) fluoresced with varying degrees of intensity (data not shown). These results were also consistent with the fact that antibodies were directed against 5'-nucleotidase itself.

Effect of unabsorbed antiserum on 5'-nucleotidase activity

When unabsorbed antiserum was added to either right-side-out vesicles or inside-out vesicles prepared from MF_2s plasma membranes, it inhibited the 5'-nucleotidase activity (Fig. 1). Similar results were obtained with vesicles prepared from MOPC 173 plasma membranes (Fig. 2). In both instances, the antiserum was more inhibitory on right-side-out than on inside-out vesicles. Incubation of membranes with equivalent amounts of non-immune serum did not affect 5'-nucleotidase activity.

Effect of the absorbed serum on 5'-nucleotidase activity present on right-side-out vesicles from MF_2s cells

The antiserum absorbed on MF_2s cells was compared first to the original complete antiserum; it was seen that its inhibitory capacity on 5'-nucleotidase activity was drastically reduced when tested on right-side-out vesicles (Fig. 3). For example, 1 µl of unabsorbed serum inhibited about 80% of the enzymatic activity, whereas the same quantity of serum after one absorption cycle inhibited about 50% and after two absorption cycles only 25–30% of the enzyme activity. In contrast, absorption of inside-out vesicles prepared from MF_2s cells had little effect on the inhibitory capacity of the antiserum (Fig. 3). Thus, the inhibitory capacity of the antibodies on right-

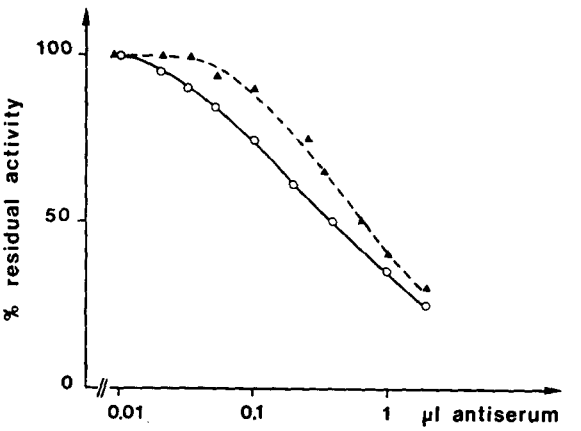

Fig. 2. Inhibition by unabsorbed anti-5'-nucleotidase antiserum of 5'-nucleotidase activity detected on right-side-out (o———o) or inside-out (▲- - - - -▲) plasma membrane vesicles from MOPC 173 cells.

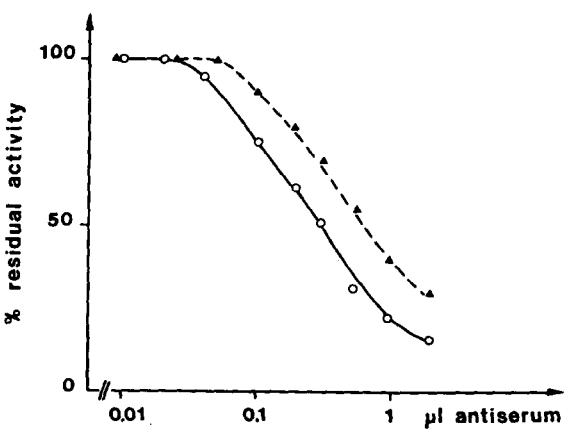

Fig. 1. Inhibition of unabsorbed anti-5'-nucleotidase antiserum of 5'-nucleotidase activity detected on right-side-out (o———o) or inside-out (▲- - - - -▲) plasma membrane vesicles from MF_2s cells.

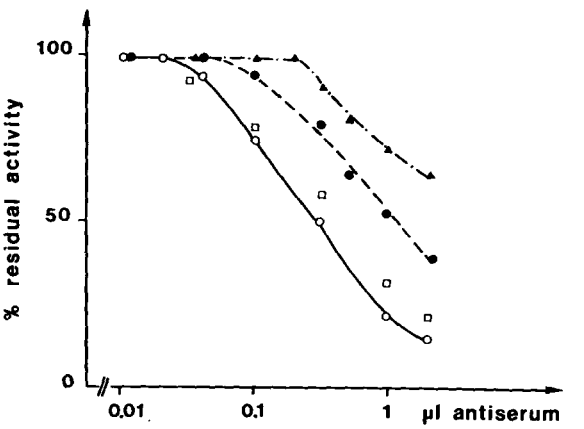

Fig. 3. Inhibition of 5'-nucleotidase activity present on right-side-out plasma membrane vesicles prepared from MF_2s cells by: o———o; unabsorbed antiserum; •- - - - -•, antiserum absorbed once on MF_2s cells; ▲- - - - -▲, antiserum absorbed twice on MF_2s cells; □, antiserum absorbed on inside-out vesicles prepared from MF_2s cells.

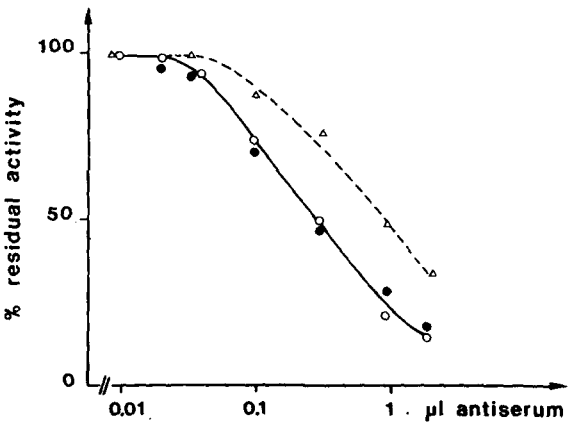

Fig. 4. Inhibition of 5′-nucleotidase activity present on right-side-out plasma membrane vesicles prepared from MF_2s cells by: o———o, unabsorbed antiserum; •, antiserum absorbed twice on P 1798 cells; △- - - - -△, antiserum absorbed twice on MOPC 173 cells.

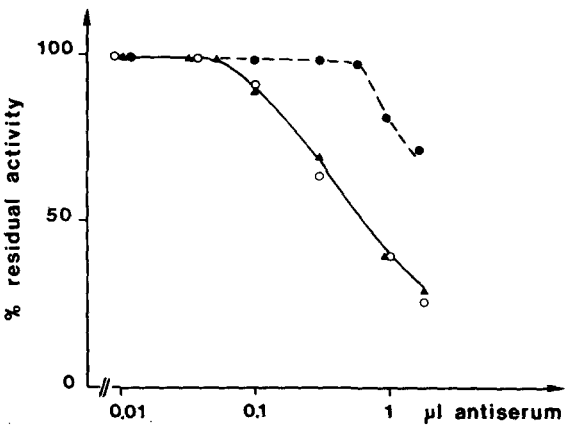

Fig. 6. Inhibition of 5′-nucleotidase activity present on inside-out plasma membrane vesicles prepared from MF_2s cells by: ▲———▲, unabsorbed antiserum; ○, antiserum absorbed twice on P 1798 cells; •- - - - -•, antiserum absorbed twice on MOPC 173 cells.

side-out vesicles was directed against external antigenic determinants which did not exist on the inner face of the plasma membrane. Two absorption cycles on MOPC 173 cells reduced the inhibitory effect of the antiserum to the level obtained after one absorbtion cycle on MF_2s cells, while no loss of activity was observed after absorption of the antiserum on P 1798 cells (Fig. 4). Thus, some antigenic determinants were shared by MF_2s and MOPC 173 cells and these appear absent from P 1798 cells.

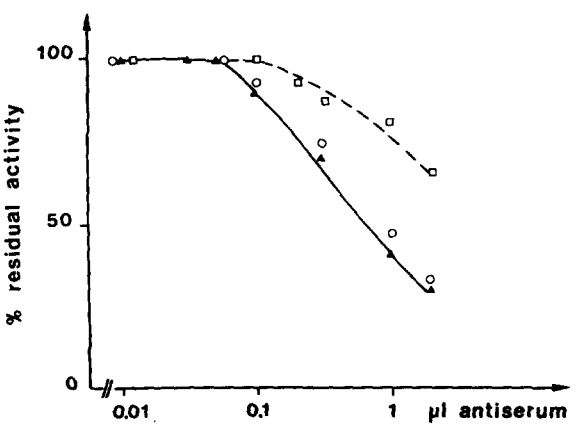

Fig. 5. Inhibition of 5′-nucleotidase activity present on inside-out plasma membrane vesicles prepared from MF_2s cells by: ▲———▲, unabsorbed antiserum; ○, antiserum absorbed twice on MF_2s cells; □- - - - -□, antiserum absorbed on inside-out vesicles prepared from MF_2s cells.

Effect of absorbed serum on 5′-nucleotidase activity detected on inside-out vesicles from MF_2s cells

Absorption on MF_2s cells did not modify the inhibitory properties of the antiserum when tested on inside-out vesicles whereas after absorption on inside-out vesicles its inhibitory activity was greatly reduced (Fig. 5). Furthermore, absorption of the antiserum on MOPC 173 cells reduced drastically the amount of specific antibodies inhibiting enzyme activity on inside-out vesicles (Fig. 6). It can be concluded that some antigenic determinants present at the cytoplasmic face of MF_2s plasma membrane are also present at the external face of MOPC 173 plasma membrane. Absorption on P 1798 cells had no effect on the inhibitory potency of the antiserum (Fig. 6).

Discussion

In the present paper we describe the inhibition of 5′-nucleotidase activity by an antiserum acting on both sides of plasma membrane prepared from either MOPC 173 murine plasmacytoma cells or the MF_2s variant. The antiserum inhibited 5′-nucleotidase activity specifically. Thus, only the 5′-nucleotidase activity among many plasma membrane-bound enzymatic activities was affected by the antiserum. Furthermore, 5′-nucleotidase purified from liver membranes was inhibited by this antiserum [12]. Finally, cells exhibiting this enzymatic activity were labelled by

fluorescent Fab fragments, whereas cells lacking the activity did not fluoresce.

The demonstration that 5′-nucleotidase activity could be inhibited from both sides of the plasma membrane points to a transmembrane disposition of the enzyme, for distinct antigenic determinants were exposed on both outer and inner membrane faces. This is consistent with the fact that 5′-nucleotidase is a membrane glycoprotein, thus oriented towards the extracellular space [4,12], and also with evidence showing it to be the anchoring protein for intracellular actin [19,20]. It is also consistent with the enzyme's carrying out a transport function [9–11]. Experiments with antisera absorbed either on MF_2s cells or on inside-out plasma membrane vesicles showed that inhibition of the 5′-nucleotidase activity at the inner or the outer face of the membrane was caused by two distinct populations of antibodies. If all or a part of the inhibition resulted from a penetration of the antibodies inside the vesicles, owing to their leaky nature, absorption of the serum on MF_2s cells, for instance, would modify not only the inhibition observed on right-side-out vesicles but also the inhibition observed on inside-out vesicles. However, it was shown that this was not the case.

The failure of P 1798 cells to absorb the antibodies was consistent with the fact that these cells lack 5′-nucleotidase activity. Furthermore, this is another demonstration of the specificity of the antiserum, in addition to the inability of fluorescent Fab fragments to label any of the thymomas lacking the enzyme.

More puzzling was the observation that absorption of the antiserum on MOPC 173 cells affected the inhibition detected on both types of vesicles prepared from MF_2s cells. This indicates that, if the catalytic site is located at the outer face of the plasma membrane of MOPC 173 cells and at the inner face of the plasma membrane of MF_2s cells, there is not a complete translocation or inversion of the enzyme. Evidence for a translocation of the catalytic site of 5′-nucleotidase in hepatic internal membranes has been demonstrated by Little and Widnell [21]. Similarly, Benedetti and Delbauffe [22] also reported that the catalytic site of 5′-nucleotidase on the plasma membrane of the hepatocyte varied according to its position on the cell surface. In the present experiments, it appears that the translocation of the catalytic site did not correspond to a complete transverse rotation of the protein, for some antigenic determinants remained in the same orientation.

In summary, we conclude that there exist at least three groups of antigenic determinants on the plasma membranes of lymphoma cells. One group is located at the external face of the membrane and may be related to the glycopeptide domain of the 5′-nucleotidase. The second group is associated with the inner face of the membrane and may represent a region of actin-5′-nucleotidase interaction. The third group of antigenic determinants is located in the vicinity of the catalytic site and varies in position with cell type.

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